<Ogirinal>Properties of Enzyme-Unhydrolyzable Residue of Lignin-Carbohydrate Complexes Isolated from Beech Wood

AUTHOR(S):

TAKAHASHI, Nobuyoshi; KOSHIJIMA, Tetsuo

Original

Properties of Enzyme-Unhydrolyzable Residue of Lignin-Carbohydrate Complexes Isolated from Beech Wood*

Nobuyoshi TAKAHASHI** and Tetsuo KOSHIJIMA**

(Received August 31, 1987)

Abstract——Water-soluble lignin-carbohydrate complexes (LCC-W) isolated from beech MWL were treated twice with commercial cellulase (ONOZUKA R-10). The unhydrolyzable fraction precipitated by enzyme treatment was divided into three fractions according to the previous report. All fractions contained neutral sugars and uronic acid. Composition and methylation analysis of sugars remaining in each fraction indicated that xylose, arabinose and galactose residues are linked to lignin possibly by benzyl ether linkage at C-2 or C-3, C-5 and C-6 positions, respectively. The results from alkaline treatment also suggested that these sugars are directly linked to lignin. The existence of alkali-stable linkage between lignin and carbohydrate also was characterized. An analysis of phenolic hydroxy group of lignin moiety suggested that phenol-glycoside linkage is not involved. The results of spectroscopic analysis indicated that lignin moiety in LCC-W consisted of typical hardwood lignin.

1. Introduction

Several reports supporting the existence of lignin-carbohydrate complexes (LCC) in plant cell walls have been published. The structural investigation of the LCC is essential for elucidating the role and biosynthesis process of this materials in living wood. Therefore, most of investigations performed on the LCC have concentrated in the clarification of the linkages between lignin and carbohydrates, and many linkage types have been proposed[1-4,4a,22]. Generally, investigations of these linkage types have been performed on the LCC, of which carbohydrate moiety previously was hydrolyzed with enzyme, having glycosidase activity. Methylation analyses of sugars remaining in the LCC after enzymatic degradation have presented significant information of these linkage types[5,6].

We have already reported that beech LCC contained alkali-labile lignin-carbo hydrate linkage[7]. Methylation of the LCC under alkaline condition such as the Hakomori method[8] does not reflect the real structure. On the other hand, it has

* Presented partly at 32nd Annual Meeting of the Japan Wood Research Society in Fukuoka (April 1982).

** Research Section of Wood Chemistry.

been reported that benzyl ether linkage of sugars with non-phenolic portion of lignin is stable in alkaline solution[9,22].

The present work describes the results of spectroscopic analysis, reducing end sugar analysis, methylation analysis and alkaline treatment of the precipitates after enzymatic degradation, and we discuss the chemical structure of beech LCC.

2. Materials and Methods

2.1 Materials and general methods

LCC was prepared from beech wood (*Fagus crenata* Bl.) by the same method described previously[7]. Infrared (i.r) spectra were taken as KBr disc using a JASCO IR-810 spectrophotometer. Ultraviolet (u.v) spectra were measured in 90% aqueous dioxane using a JASCO UVDEC-420 spectrophotometer. Nuclear magnetic resonance spectra were obtained on a Varian XL-200 NMR spectrometer at 20°C, except for xylan at 80°C. ^{1}H-NMR spectra were obtained in d-chloroform after acetylation of samples, and ^{13}C-NMR spectra in d-DMSO and D_2O. Conductometric titration was performed by using a TOA digital conductometer CM-15A. Gel-filtration was performed on a column of Sephadex LH-60, using 50% aqueous dioxane as an eluent. Gas-liquid chromatography (g.l.c) and g.l.c-m.s were conducted with Shimadzu GC-4CM and LKB-9000 system.

2.2 Enzymatic degradation

The LCC-W isolated from beech wood was treated with commercial cellulase (ONOZUKA R-10) for 48 hrs at 37°C in 0.05 *M* acetate buffer (pH 4.6). The concentrations of the substrate and enzyme used were 1.0% and 0.1%, respectively. The enzyme used was purified in advance by gel-filtration on Sephadex G-50 to remove lower molecular weight fractions. The unhydrolyzable fraction precipitated by enzyme treatment was collected by centrifugation and re-treated with enzyme under the same condition. The precipitate was washed with water to remove the water-soluble materials and the residue was lyophilized (B-P).

2.3 Fractionation of the precipitate (B-P)

As shown in a scheme of Fig. 1, the precipitate obtained by enzyme treatment was dissolved in a small amount of 50% aqueous dioxane and the solution was poured into a large volume of ethanol, then the supernatant and the residue were separated by centrifugation to obtain ethanol-soluble fraction (P-III). The residue was dissolved in the solution of pyridine-acetic acid-water (9:1:4) and divided into the chloroform extract (P-I) and the residue (P-II).

2.4 Chemical analyses

Lignin content was determined by acetyl bromide method[10]. Carbohydrate

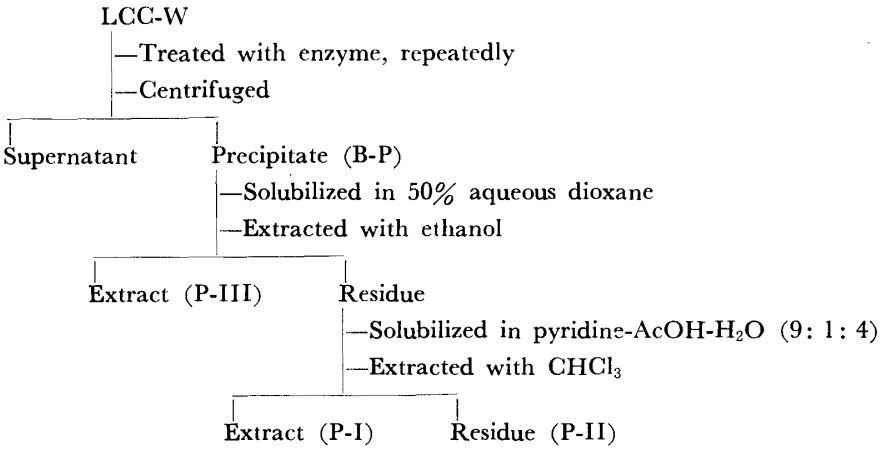

Fig. 1. Fractionation of precipitate remaining after enzymatic degradation.

content and composition were determined by g.l.c of alditol acetates on a column of 3% ECNSS-M after hydrolysis of LCC with 2 *M* trifluoroaceticc acid (TFA) for 1 hr at 121°C followed by conversion of the component sugars to corresponding alditol acetates[11]. Uronic acid content was measured by colorimetric determination using a cysteine-harmine reagent[12]. Phenolic hydroxy group was calculated by $\Delta\varepsilon_i$ method (A)[13], conductometric titration (B)[14], aminolysis with pyrrolidine (C)[15] and ^{1}H-NMR method (D)[16]. Reducing end sugar was determined as follows. Sample was reduced with sodium borohydride, and then hydrolyzed with 2 *M* TFA. The hydrolysate was applied to a Dowex 1×8 (OH^-) column to obtain sugar alcohol[17]. The sugar alcohol eluted with water was determined by g.l.c after its conversion to corresponding acetates.

2.5 Alkaline degradation

Each precipitate fraction was treated with 1.0 *N* sodium hydroxide containing sodium borohydride for 2 hrs at ambient temperature. After neutralization with acetic acid, the resulting reaction mixture was divided into water soluble fraction (S) and insoluble fraction (R) by centrifugation.

2.6 Methylation analysis

B-P and B-P_P, of which phenolic hydroxy group was protected as described previously[14], were methylated by the Hakomori method[8]. The partially methylated alditol acetates obtained from conventionally methylated samples were analyzed by g.l.c on columns of both 3% OV-225 and 3% ECNSS-M, and by g.l.c-m.s on a column of 3% OV-225.

3. Results and Discussion

3.1 Isolation and fractionation of B-P

The LCC-W was treated with enzyme to obtain the lignin-rich LCC (B-P).

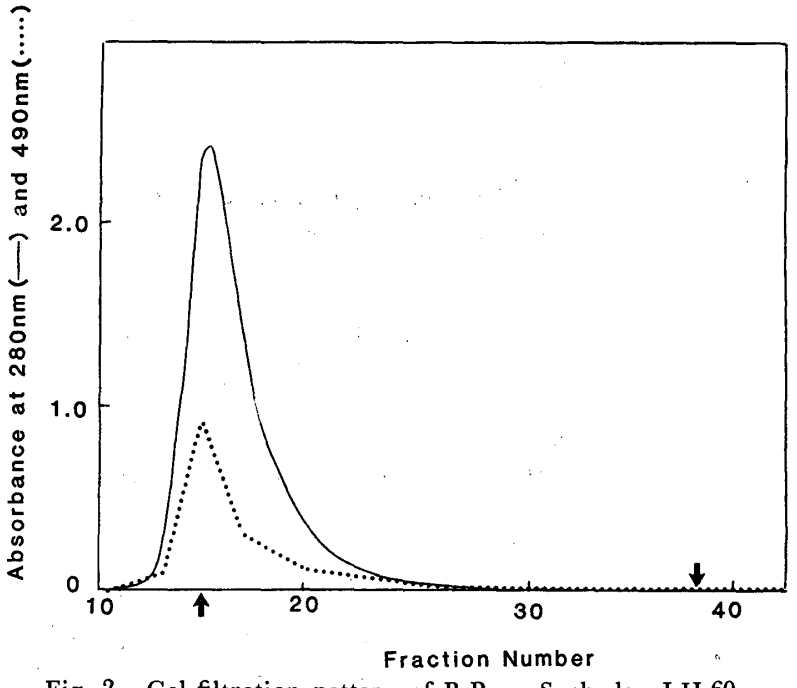

Fig. 2. Gel filtration pattern of B-P on Sephadex LH-60. Two arrows represent the void volume and the elution volume of xylose, respectively.

The B-P fraction was applied to gel-filtration on a Sephadex LH-60 column (Fig. 2). Gel-filtration curve showed only one peak consisting of the eluting fractions of sugar and lignin overlapped each other. The peak was eluted at void volume, as shown in the case of Sephadex LH-20 column[18]. This result showed that B-P did not contain not only mono- and oligosaccharides but also lignin of lower molecular weight and LCC, inspite of the extensive degradation of carbohydrate chain. Most of lignin moiety in the original LCC-W was detected in B-P fraction (Table 1). These results suggest that lignin moiety in LCC-W from beech wood consists of lignin having a relative higher molecular weight.

Table 1. Chemical composition of fractions precipitated after enzyme treatment

	Yield[a)]	Lignin[b)]	Neutral sugar[b)]	Uronic acid[b)]
B-P	15.0	80.9	2.9	3.5
P-I	9.1	85.7	0.7	3.4
P-II	9.2	70.0	5.4	3.7
P-III	4.3	76.4	2.2	2.5
LCC-W	100	16.2	63.4	12.2

a) Percent of the original LCC, b) Percent of each fraction.

Therefore, on the basis of the difference of carbohydrate content[19], the B-P was divided into three fractions (see experimental).

3.2 Chemical analysis

As shown in Table 1 and 2, all fractions contained neutral sugar and uronic acid. On comparison with the analytical data of the original LCC-W, the decrease of contents of uronic acid and xylose in each fraction suggest that uronic acid in LCC-W was not derived from pectic substance but from glucuronoxylan, since no esterase activity in the enzyme used was found[20]. The neutral sugar and uronic acid residues remaining in each fraction, especially xylose, arabinose and galactose residues could be linked covalently lignin moiety since these sugars remain in a large amount (Table 2).

Table 2. Neutral sugar composition of the precipitated fractions (mole %)

	Rha	Ara	Xyl	Man	Gal	Glc
B-P	2.7	13.0	31.3	11.2	31.0	6.3
P-I	8.9	6.8	52.9	6.8	40.4	7.6
P-II	11.9	9.7	37.0	7.6	27.3	6.5
P-III	17.6	25.8	31.8	3.2	15.5	6.0
LCC-W	2.4	1.8	86.5	2.4	2.4	4.3

3.3 Phenolic hydroxyl content in each fraction

Enoki *et al.*[21] reported that all of the glycosidic lignin-carbohydrate bonds in LCC model compounds were hydrolyzed by glycanase enzyme. Yaku *et al.*[22] reported that a lignin of low molecular weight was liberated from pine LCC by the enzymatic degradation. Hayashi[23] reported the existence of glycosidic linkage with phenolic hydroxyl groups of lignin in wheat straw. Hence, we studied on the possibility of phenol-glycosidic linkage in LCC-W in comparison between phenolic hydroxyl contents before and after treatment of LCC-W with glycosidase enzyme. As for the determination of phenolic hydroxyl content, four different methods were used. The values obtained by these methods are somewhat different each other. However, the relative values between B-P and LCC-W obtained from these methods could be applicable to this purpose (Table 3). In the case of method A and B, the values per lignin content of B-P were approximately identical with those of the original LCC-W. Actually, there was no remarkable difference between B-P and the original LCC-W in the values measured by method D. However, the data from method C showed large differences not only between B-P and LCC-W but also in those from method A and method B, especially in the value of LCC-W rich in carbohydrate. The larger value obtained from method C in comperison wih ones from other methods is based on the result that acetyl groups substituted at anomeric carbon of reducing end sugar in carbohydrate moiety as well as phenolic acetyl groups are deacetylated with pyrrolidine faster than those at other

Table 3. Phenolic hydroxyl content of each fraction

	A		B		C		D
	I	II	I	II	I	II	
B-P	2.62	3.24	2.76	3.41	3.18	3.93	0.27
P-I	2.77	3.23					
P-II	2.53	3.61					
P-III	2.94	3.82					
LCC-W	0.51	3.15	0.58	3.58	2.70	16.7	0.28

A: Values determined by $\Delta\varepsilon_i$ method. B: Values obtained by conductometric titration. C: Values determined by aminolysis with pyrrolidine. I; Percent of each fraction. II; Percent of lignin content. D: Values calcd. per OMe content determined from ^{1}H-NMR.

carbons[24]. The results from method C indicate that this methods is not uitable for the determination of phenolic hydroxyl groups in the substance containtng sugars such as LCC. All results except for the data from method C showed that enzymatic degradation of the LCC-W did not result in a increase in phenolic hydroxyl groups. This result suggests that there are no phenol-glycoside linkage in LCC-W, coinciding with Iversen's report[5].

3.4 Spectroscopic analysis

The u.v spectra of all fractions (Fig. 3) were similar to those of original LCC-W, which showed a maximum absorption at 279 nm[7]. The absorption coefficient

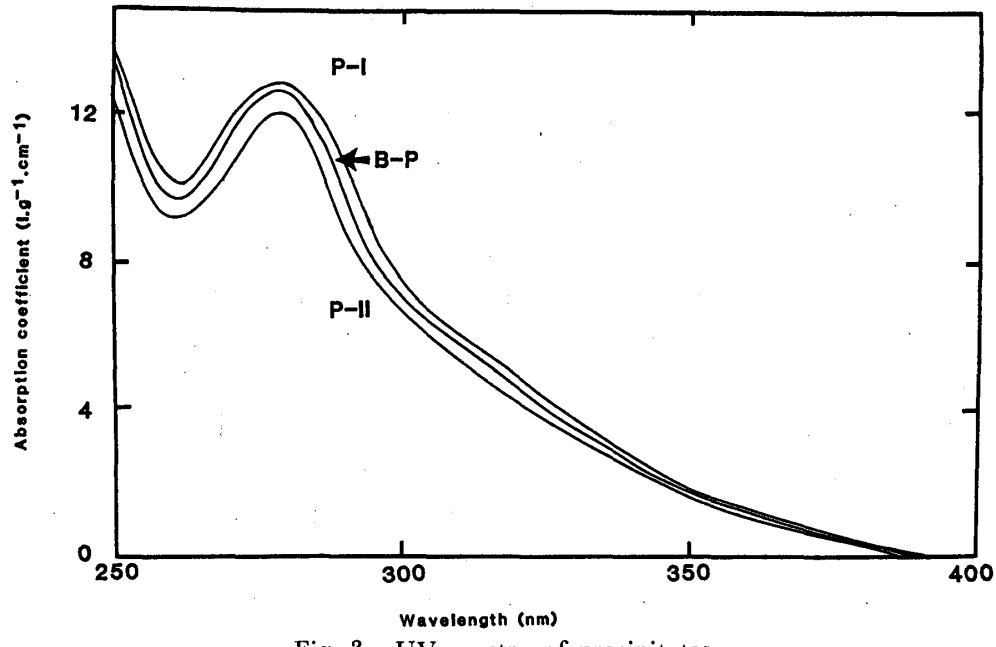

Fig. 3. UV spectra of precipitates.

at 279 nm for B-P, P-I and P-II were calculated to be 12.5, 12.7 and 12.0 $l.g^{-1}.cm^{-1}$, respectively, and these values were identical with the value reportd for MWL of beech[25]. These results suggest that lignin moiety of LCC consisted of lignin similar to MWL. Kato *et al.*[26] reported that the u.v spectra of LCC containing *p*-hydroxycinnamates and ferulates had a maximum at 312-320 nm. The u.v spectra obtained in the precipitate fractions of beech wood did not give a maximum at

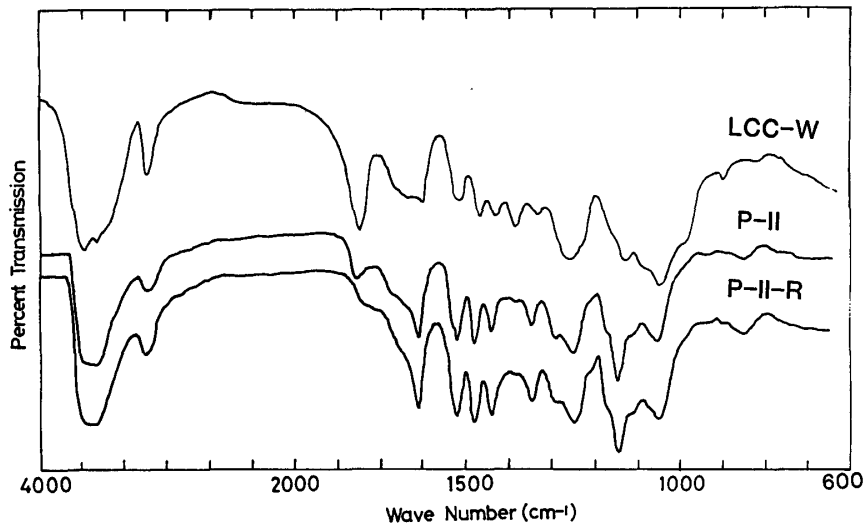

Fig. 4. IR spectra of precipitates and LCC-W.

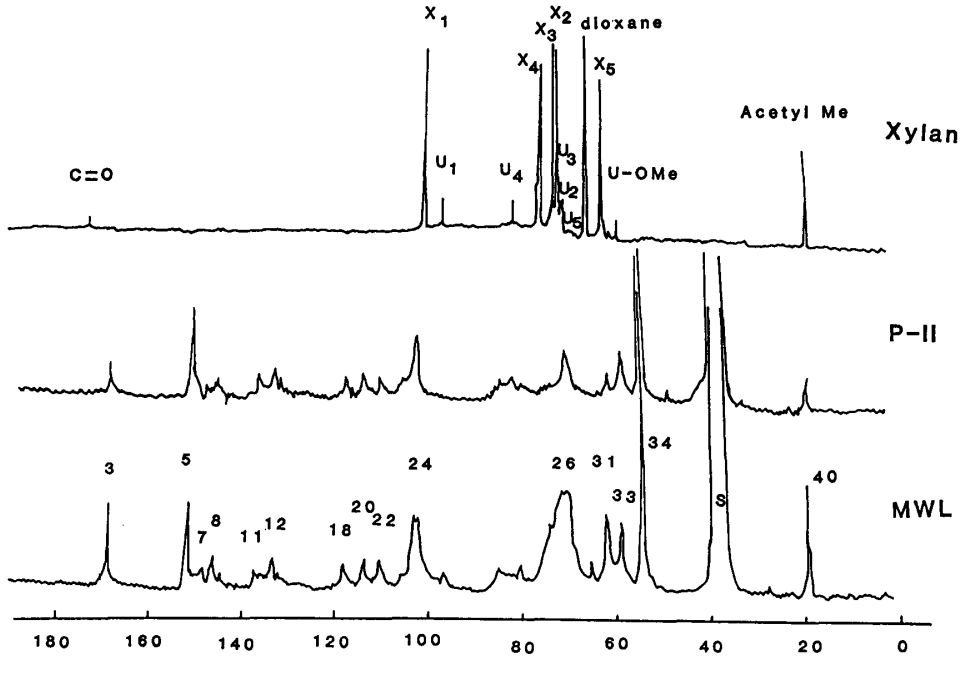

Fig. 5. ^{13}C-NMR spectra of xylan, P-II and MWL. X_1-X_5 and U_1-U_5 represent carbon number of xylose and uronic acid, respectively.

this band, indicating that beech LCC-W does not contain *p*-hydroxycinnamic and ferulic acids. Figure 4 shows i.r spectra of the original LCC-W, P-II, and P-II-R which is water-insoluble fraction after alkaline treatment. All fractions showed typical i.r spectra of hardwood lignin. The strength of the absorbance at 1,730 cm^{-1} due to esterified carbonyls was in accordance with sugar and uronic acid content (Table 1 and 6), suggesting that this absorbance is based on acetyl groups and uronic ester in xylan[14]. ^{13}C-NMR spectra are shown in Fig. 5. The assignment of signals in MWL was made by reference to the data of Ludemann *et al*[27]. The ^{13}C-NMR spectrum of P-II showed peaks of No. 3 and No. 40 due to acetyl groups in xylan, and No. 5 and No. 26 peaks indicating to be hardwood lignin. The results from ^{13}C-NMR spectra also showed that lignin moiety in LCC-W consists of lignin similar to MWL.

3.5 Reducing end sugar of B-P

Sugar chain in LCC has been investigated by the determination of reducing end sugar and methylation analysis[5,6]. Reducing end sugars of LCC-W and B-P were estimated by conversion of the component monosaccharides to the corresponding alditols with sodium borohydride. The results are given in Table 4. Beside the absence of phenol-glycoside, if the other glycosidic linkages do not exist between lignin and carbohydrate[5], the apparent sugar chain lengths in LCC-W and B-P are calculated to be 53 and 4.4, respectively, from the data obtained. These values may indicate that one-fourth to one-fifth of sugar residues remaining in B-P take part in the linkage to lignin. But these values do not reflect a real chain length since xylan has a side chain. On the other hand, weight percent of reducing end sugar after enzymatic degradation increased in all component sugars except for rhamnose, indicating that enzymatic degradation takes place at several positions of sugar chain. Especially, arabinose residue was found only after enzymatic degradation. This result which is different from the data of softwood LCC[3,6] implies that beech LCC-W does not contain arabinan.

Table 4. Reducing end sugar of B-P and LCC-W fractions

	Total	Rha	Ara	Xyl	Man	Gal	Glc
B-P	22.6	0	30.9	18.9	40.0	29.9	34.5
LCC-W	1.9	0	0	1.6	5.6	9.8	3.7

Values are expressed as weight percentages of each component sugar.

3.6 Alkaline degradation

It is well known that alkali-labile linkages between lignin and carbohydrates exist in LCC. We have recently reported the presence of two different types of

Table 5. Alditols released in the water-soluble fraction (S) from alkaline treatment

Component	Alditols[a)]					
	Rha	Ara	Xyl	Man	Gal	Glc
P-I-S	—	trace	0.02	—	trace	—
P-II-S	—	trace	0.13	—	trace	—
P-III-S	—	trace	0.07	—	trace	—

a); Milligram per 100 mg of the original P-I, P-II and P-III.

alkali-labile linkages in beech LCC[14,18]. To elucidate this type of linkage in the precipitate fractions after enzymatic degradation, the precipitates were subjected to alkaline degradation and the alditols were analyzed by g.l.c (Table 5). The alditols released by alkaline treatment are thought to be linked directly to lignin moiety. Arabinitol, xylitol and galactitol were detected. Especially xylitol was prominent, indicating that the main sugar chain linked lignin is xylan. On the other hand, a small amount of sugar still remained in all water-insoluble fractions (R) after alkaline treatment, and P-I-R and P-II-R fractions no longer contained uronic acid (Table 6). These results indicated the possibility that B-P also contains uronic ester and alkali-stable linkage.

Table 6. Summative chemical composition of residues from alkaline treatment

Component	Yield[a)]	Uronic acid[b)]	Neutral sugar[b)]	Neutral sugar composition					
				Rha	Ara	Xyl	Man	Gal	Glc
P-I-R	89	trace	0.5	6.7	10.4	43.0	6.3	25.9	8.6
P-II-R	82	trace	2.0	4.3	15.3	39.0	4.8	26.4	10.2
P-III-R	80	1.2	1.4	0.3	3.1	75.2	1.1	4.2	16.2

a); Percent of the original residue. b); Percent of each fraction.

3.7 Methylation analysis

The methylated sugars identified from B-P and B-P_P are shown in Table 7. The predominant methylated xyloses indicated that the backbone structure of the remaining sugar in B-P comprised of 1→4 linked xylan, in agreement with the results of reducing end sugar analysis and sugar composition. The identification of 2 or 3-O-Me-Xyl and 2,3-di-O-Me-Ara indicated the possibility that lignin moiety is linked at C-2 or C-3 position of xylose residue and at C-5 position of arabinose residue, because the absence of arabinan is anticipated from the results of reducing end sugar analysis. The detection of 2,3-di-O-Me-Gal suggested the linkage to lignin at C-6 position on galactose residue, since it is known that etherification at primary hydroxy groups is preferred[28].

Table 7. Results from methylation analyses of B-P and B-Pp fractions

Identified sugars[a)]	T[b)]	B-P[c)]	B-P[c)]
2,3,4,6-Gal	1.31	2.3	2.0
2,3,6-Gal	2.49	9.2	9.8
2,3,5-Ara	0.51	5.3	2.3
2,3,4-Xyl	0.70	15.1	13.7
2,3-Ara	1.35	8.4	11.4
2,3-Xyl	1.54	38.2	33.1
2,3-Gal	5.63	5.4	7.0
2 or 3-Xyl	2.97	16.1	19.7

a) 2,3,4,6-Gal represents 2,3,4,6-O-methyl-D-galactitol, and so on. b) Retention times are relative to that of 2,3,4,6-Glc on 3% ECNSS-M column. c) Values are expressed as relative molar percentages of the total identified-methylated sugars.

We have already reported that benzyl ether and ester linkages, which are unstable for alkaline solution, exist between lignin and carbohydrate moiety in beech LCC[14,18]. Therefore, methylation of the LCC under alkaline condition such as Hakomori method[8] does not reveal the real structure of sugar chain. However, it has been known that non-phenolic benzyl ether is stable even under alkaline condition[9]. The results of methylation analysis of phenol-protected material (B-Pp) were compared with those of originals (B-P) with free phenolic hydroxyl groups. If the benzyl ether linkage to carbohydrate exist in B-P, this comparison reveals differences in amounts of methylated alditols. The data of B-P were different from those of B-Pp in amount of 2,3,5-tri-O-Me-Ara, 2,3,4-tri-O-Me-Xyl, 2,3-di-O-Me-Gal 2,3-di-Me-Ara, 2,3-di-O-Me-Xyl and 2 or 3-O-Me-Xyl. Especially, the amounts of 2 or 3-O-Me-Xyl and 2,3-di-O-Me-Ara in B-Pp increased, and the amounts of 2,3,4-tri-O-Me-Xyl, 2,3-di-O-Me-Xyl and 2,3,5-tri-O-Me-Ara decreased consistently, indicating that xylose and arabinose participate in lignin-carbohydrate linkage, and xylose residues are linked to lignin at C-2 or C-3 position, arabinose at C-5 position. And the results also indicate that the methylation under alkaline condition is not suitable for the structural analysis of the LCC containing alkali-labile linkage such as benzyl ether of phenolic moiety of lignin. The results from alkaline treatment and methylation analysis indicated that these sugars are linked to lignin throuh the benzyl ether linkage.

References

1) A. Chesson, A.H. Gordon and J.A. Lomax: J. Sci. Food Agri., **34**, 1330-1340 (1983).
2) J.-P. Joseleal and R. Kesraoui: Holzforschung, **40**, 163-168 (1986).
3) J.L. Minor: J. Wood Chem. Technol., **2(1)**, 1-16 (1982).
4) M. Remko: Holzforschung, **40**, 205-209 (1986).

4a) T. WATANABE, S. KAIZU and T. KOSHIJIMA: Chem. Lett., 1871-1874 (1986).
5) J.L. MINOR: J. Wood Chem. Technol., **6**(2), 185-201 (1986).
6) T. IVERSEN: Wood Sci. Technol., **19**, 243-251 (1985).
7) J. AZUMA, N. TAKAHASHI, M. ISAKA and T. KOSHIJIMA: Mokuzai Gakkaishi, **31**, 587-594 (1985).
8) S. HAKOMORI: J. Biochem., **55**, 205-208 (1964).
9) B. KOŠÍKOVÁ, D. JONIAK and L. KOSÁKOVÁ: Holzforschung, **33**, 11-14 (1979).
10) D.B. JOHNSON, W.E. MOORE and L.C. ZANK: Tappi, **44**, 793-798 (1961).
11) P. ALBERSHEIM, D.J. NEVINS, P.D. ENGLISH and H. KARR: Carbohydr. Res., **5**, 340-345 (1967).
12) A.H. WARDI, W.S. ALLEN and R. VARMA: Anal. Biochem., **57**, 268-273 (1974).
13) N. TERASHIMA: "Chemistry of lignin", ed. Nakano, Uni-pub. Co., p.131-132 (1979).
14) N. TAKAHASHI and T. KOSHIJIMA: Wood Sci. Technol., submitted for publication.
15) P. MÅNSSON: Holzforschung, **37**, 143-146 (1983).
16) C.H. LUDING, B.J. NIST and J.L. MCCARTHY: J. Am. Chem. Soc., **86**, 1186-1202 (1964).
17) H. YAMAGUCHI, S. INAMURA and K. MAKINO: J. Biochem., **79**, 299-303 (1976).
18) N. TAKAHASHI and T. KOSHIJIMA: Woos Sci. Technol., submitted for publication.
19) K. LUNDQUIST and R. SIMONSON: Svensk Papperstidning, **11**, 390 (1975).
20) A. KATO, J. AZUMA and T. KOSHIJIMA: Chem. Lett., 179-140 (1983).
21) A. ENOKI, F. YAKU and T. KOSHIJIMA: Holzforschung, **37**, 135-141 (1983).
22) F. YAKU, Y. YAMADA and K. KOSHIJIMA: Holzforschung, **30**, 148-156 (1976).
23) A. HAYASHI: Nippon Nogei Kagakukaishi, **35**, 83-85 (1961).
24) unpublished data.
25) N. TERASHIMA: "Chemistry of lignin", ed. Nakano, Uni-pub. Co., p. 175 (1979).
26) A. KATO, J. AZUMA and T. KOSHIJIMA: Holzforschung, **38**, 141-149 (1984).
27) H.-D. LUDEMANN und H. NIMZ: Makromol. Chem., **175**, 2409-2422 (1974).
28) G.J. LEARY, D.A. SAWTELL and H. WONG: Holzforschung, **37**, 11-16 (1983).